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Effect of nitric oxide donor nitroglycerin on bone mineral density in a rat model of estrogen deficiency-induced osteopenia

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Abstract

Nitric oxide (NO) may modulate estrogen's anabolic effects on bone homeostasis by restraining osteoclast-mediated bone resorption and stimulation of osteoblast activity. Accordingly, NO donated by organic nitrates, including nitroglycerin, is thought to protect against bone loss associated with estrogen deficiency. In this study, we have explored this phenomenon. Thirty-two 12-week-old female Wistar rats were divided into four groups prior to bilateral ovariectomy or a sham operation. The ovariectomised rats received (1) vehicle control (OVX control), (2) 17-β-estradiol (OVX+E2), or (3) transdermal nitroglycerin (OVX+NG) for 4 weeks. Femoral and tibial bone mineral density (BMD), serum alkaline phosphatase and urine deoxypyridinoline and NO metabolites were analysed at the end of the study period together with failure torque and torsional rigidity of the tibiae and cellular localisation of the NO-synthase (NOS) isoforms. In OVX+E2 group, proximal and distal femoral and proximal tibial BMD exceeded that of the Sham controls. Nitroglycerin prevented BMD loss at these three sites at levels comparable to that of the Sham controls. Deoxypyridinoline excretion did not change except in the OVX-E2 group that showed an expected reduction when compared to the Sham and OVX controls. There were no treatment-related differences in total alkaline phosphatase or urinary NO metabolites. Tibial failure torque was comparable between the groups but both OVX+E2 and OVX+NG groups showed decreased torsional rigidity compared with the OVX controls. Endothelial and inducible NOS were found in osteoblast-like cells associated with calcifying cartilage spicules in the distal femoral metaphysis. These data confirm previous findings and show that nitroglycerin counteracts the estrogen deficiency-induced osteopenia in the ovariectomised rat model. Organic nitrates may thus be beneficial in conditions where bone turnover is compromised such as in osteoporosis.

Keywords: Bone mineral density; Estrogen; Nitroglycerin; Nitric oxide; Torsional stiffness; Torsional strength

Introduction

Nitric oxide (NO) has emerged as a potent regulator of bone metabolism mediating the effects of proinflammatory cytokines [1–4] estrogen [5,6], mechanical strain [7,8], fluid flow, and flow-induced shear stress [9–11]. NO metabolites decrease in postmenopausal and other amenorrhoeic women and are increased by estrogen replacement [12–15]. During menstrual cycle, blood NO metabolite concentrations correlate with estrogen levels, being higher in the follicular than in the secretory phase [16]. Targeted disruption of the eNOS gene in mice results in marked reduction in bone volume, bone formation rate, and bone mineral density

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(BMD) [17,18]. The function of the eNOS gene-deficient osteoblasts was restored by administration of exogenous NO donor, but their response to 17β-estradiol was blunted both in vitro and in vivo [6,17,18].

These findings imply that NO donors might be useful in the prevention and treatment of osteopenic conditions. Wimalawansa and co-workers have shown that transdermal nitroglycerin (NG) prevents bone loss in (i) a rat model of estrogen-depletion induced osteopenia [19–21]; (ii) a rat model of prednisolone-induced osteopenia [22], and (iii) women after ovariectomy [23]. In ovariectomised women, NG was as efficient as estrogen and seemed to suppress osteoclast-mediated resorption (urinary *N*-telopeptides) and increase osteoblast activity (alkaline phosphatase and osteocalcin) [23]. These data suggest that the effects of estrogen on bone metabolism may be, at least in part, NO dependent and, reversely, that organic nitrates may be beneficial in prevention of excessive loss of bone in postmenopausal women.

In the present investigation, we aimed to confirm and extend the efficiency of NG therapy in prevention of estrogen deficiency-induced loss of femoral and tibial BMD. In addition, we aimed to determine the influence of such treatment on tibial torsional failure torque and torsional rigidity and the effect of estrogen manipulation on cellular expression levels of the three NOS isoforms.

Materials and methods

Animals

Thirty-two nulliparous 12-week-old female Wistar rats with a mean body weight range of 160–180 g were studied (Tuck and Son, Battlebridge, Essex, UK). Four rats were housed per cage with 12-h light/12-h dark cycle at a 22 ± 2°C temperature range. The weight of pellet diet to ovariectomised rats was restricted to the food weight consumed by the control Sham-operated animals. The pelleted diet (RM1 maintenance diet, Special Diet Services, Witham, U.K.) contained 0.71% calcium, 0.5% phosphorus, 600 IU/kg vitamin D_3, and 0.95% arginine. All experiments were approved by the institutional review board and the principles of Declaration of Helsinki were followed.

Surgical procedures

Twenty-four rats were anaesthetised with Isoflurane (Rhone-Merieux, Essex, UK) inhalation and underwent a bilateral ovariectomy (OVX) via dorsal incision. Ovaries were excised and oviducts replaced, with minimum disruption to surrounding soft tissues, and the incisions were closed with Michele clips. In addition, eight animals underwent a sham surgical procedure where the presence of both ovaries was confirmed and incisions closed in an otherwise identical manner. Prior to the surgical procedure, animals were allocated, by body weight, to four different study groups (Sham, OVX, OVX+E2, and OVX+NG; $n = 8$ per group).

Treatment regiments and tissue preparation

Sham-operated and ovariectomised controls received 250 μl vehicle control comprising 95% v/v corn oil and 5% v/v ethanol subcutaneously. 17-β-estradiol (E2) was administered subcutaneously in a constant dosing volume of 250 μl at 30 μg/kg body weight per day, dissolved in the above-mentioned vehicle. NG (1 mg of 2% w/w ointment (Cusi (UK) Ltd., Haselmere, U.K.), was applied twice a day to alternative sides of shaved left and right hind limb dorsal sites (1.5 cm^2 area). From Day 1 postoperation, animals received their treatment for 28 consecutive days. Welfare of the animals was carefully examined and confirmed at least twice a day. All animals were weighed once a week, then killed at Day 28 by exsanguination under carbon dioxide-induced anaesthesia. The success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. Femora and tibiae were removed, cleaned, and placed in 10% v/v neutral buffered formalin. After 48 h, the tissues were thoroughly rinsed in distilled water and stored in phosphate-buffered saline (PBS) with sodium azide. After DEXA scanning, the femora were decalcified (for immunocytochemistry) with frequent rotation in 0.1 M Tris (pH 6.95) containing 10% w/v ethylenediaminetetracetate (EDTA) disodium salt (Sigma-Aldrich Chemical Co., Poole, UK) and 7.5% w/v polyvinylpyrrolidone (MW 40,000; Sigma-Aldrich) at 4°C. The decalcification solution was replaced every 3 days, and when the demineralisation process was complete according to X-rays, the femora were washed and cleared of any visibly remaining mineral salt deposits and stored in 30% v/v ethanol at 4°C before dehydration and paraffin embedding.

Deoxypyridinoline, NO metabolites, and total alkaline phosphatase

At the end of the experiment, urine samples were collected over a 16-h period from rats housed individually in metabolic cages with free access to water but restricted amount of food. Due to the limited number of cages, urine samples were collected only from four randomly selected animals per group. Urinary deoxypyridinoline was measured using an automated high-performance liquid chromatography (HPLC) on-line solid phase extraction procedure [24]. For NO assay, urine samples were deproteinised by cold ethanol precipitation. NO was detected by the chemiluminescence reaction of NO with ozone generated from pure oxygen using a Sievers NOA 270B NO analyser (Sievers Instruments, Boulder, CO). Fifty-microliter samples were injected into the purge vessel where NO_2^- and NO_3^- were converted to NO in a reducing mixture of 0.1 M VCl_3

in 1.0 M HCl at 87 ± 0.5°C. Standard curves were generated by using known concentrations of sodium nitrate. Urinary creatinine levels were measured using a colorimetric method based on Jaffe reaction and both deoxypyridinoline and NO_2^-/NO_3^- values were corrected for 16-h urinary creatinine. For serum samples, the rats were fasted overnight before blood samples were collected from the abdominal aorta, followed by centrifugation and storage at −20°C. Serum total alkaline phosphatase (ALP) was assayed using a Sigma Diagnostics[R] Alkaline Phosphatase Kit with *p*-nitrophenol as a standard. Serum total protein was measured using a modified Bradford's protein assay to correct the ALP values for total protein. All urine and serum analyses were performed in triplicates.

Bone mineral density (BMD) measurement by dual energy X-ray absorptiometry (DEXA)

Bone mineral density was assessed by DEXA using a Hologic QDR-1000 X-ray bone densitometer (dual X-ray source of 70 and 140 kVp) and an ultrahigh resolution software program with 0.0254 cm line spacing and a point resolution of 0.0127 cm. Femora (n = 8 per group) and tibiae (n = 7 per group) were analysed in 3 cm depth of water to simulate a standardised proportion of surrounding soft tissue. Scan analysis was completed by using the "compare" feature in order to ensure consistency of placement of the region of interest. The region of interest box size was kept constant for all femoral and tibial sites. The BMD coefficient of variation (CV) was calculated by repeat scanning for six times with and without sample repositioning between scans. BMD was measured from the proximal, midshaft, and distal femur and tibia in eight and seven animals per group, respectively.

Mechanical testing of tibial failure torque and torsional rigidity

A torsion test was used to measure the mechanical properties of the tibiae (n = 7 per group). Torque was measured with a custom fabricated gage utilising strain gages and rotational displacement with a torsional encoder (resolution 0.25 degrees). The data were logged with an 11 channel, 10 bit analogue to digital card (ADC-11, Pico Technology, Cambridgeshire, UK). The computers were cross-calibrated for testing of each batch, using standard weights at known moment arms and applied to the torsion gage. Individual calibrations of clockwise and anticlockwise torque were performed for testing groups of left or right tibiae so that both samples were tested with internal rotation (i.e., distal epiphysis rotates internally compared to proximal epiphysis).

The tibiae were cleaned of soft tissues, immersed in normal saline, and kept moist throughout the test procedures. The length and width of tibiae were measured with sliding callipers (accuracy ± 0.5 mm). The length was measured from the intercondyloid fossa (proximal) between the lateral and medial maleolus (distal) and width taken at the interosseous crest from lateral to medial aspect. The midpoint of tibial diaphysis was marked and the epiphyseal parts were cut off 15 mm distal and 10 mm proximal to the midpoint so that standardised lengths of diaphysis were potted in testing cups that were axially aligned on a jig and positioned at the same point. The samples were potted in a low melting point Cero Low Alloy (MCP-47, Mining and Chemical Products, Northants, UK), which start to melt at temperatures below which collagen degrades. The tibiae were internally rotated at a pseudostatic rate (360 degrees per minute) to limit the effects of rate related damping. Applied torque and angular displacement curves were generated and the following mechanical characteristics were recorded: (i) the failure torque (the failure point was defined as a decrease in moment with increasing angular deformation; Nm) and (ii) the torsional rigidity (defined as the ratio of applied torque to resultant angle of deformation in the linear region of the curve; Nm/degree). Because the fractures occur at diaphyseal midshaft positions, these mechanical analyses reflect primarily the effect of the treatment on cortical bone.

Nitric oxide synthase antibodies

Monoclonal antibodies against neuronal NOS (nNOS) were raised against a 22.3 kDa protein fragment corresponding to amino acids 1095–1289 of human nNOS protein sequence. Polyclonal antibodies to inducible NOS (iNOS) were raised against a 21 kDa protein fragment corresponding to amino acids 961–1144 of mouse macrophage iNOS sequence. Polyclonal antibodies to eNOS were raised against a 20.4 kDa protein fragment corresponding to amino acids 1030–1209 of human eNOS sequence. All antibodies were obtained from Transduction Laboratories (Lexington, KY) and were cross-reactive with the corresponding rat NOS isoforms determined by Western blots as previously reported [25].

Immunocytochemistry of NOS isoforms

Sections of 3-μm thickness of decalcified and paraffin embedded femora were mounted onto APES-coated slides,

Table 1
Effect of the treatments on cumulative mean body weight gain

Day	Sham	OVX	OVX + E2	OVX + NG
7	13.4 ± 1.2[b]	23.1 ± 1.5[a]	8.1 ± 2.5[b]	3.9 ± 1.2[a,b]
14	24.5 ± 2.5[b]	47.6 ± 2.3[a]	19.1 ± 2.8[b]	22.0 ± 1.7[b]
21	36.0 ± 3.3[b]	74.3 ± 3.7[a]	29.4 ± 3.1[b]	38.4 ± 2.5[b]
28	44.0 ± 3.5[b]	88.9 ± 4.1[a]	38.9 ± 2.8[b]	63.4 ± 3.5[a,b]

Note. Cumulative weight gain over 28-day treatment period (n = 8 per group) expressed as grams ± SE. Statistically significant differences indicated by [a] $P < 0.001$ vs. Sham control; [b] $P < 0.001$ vs. OVX control.

Table 2
Urine and serum biochemical assays

Parameter	Sham	OVX	OVX + E2	OVX + NG
Dpd (nM)	126.0 ± 22.1	135.0 ± 9.7	73.40 ± 2.4[a,b]	126.8 ± 8.2
Total ALP (μM)	532.4 ± 26.15	543.9 ± 12.94	514.7 ± 18.28	577.7 ± 11.12
NO_2^-/NO_3^- (μM)	533.8 ± 236.3	573.9 ± 195.5	1020.0 ± 74.7	735.0 ± 113.5

Note. Dpd and NO_2^-/NO_3^- measurements were from 16-h urine collection ($n = 4$ per group) and the values were corrected for creatinine concentration (mM). Total ALP was from overnight fast serum ($n = 8$ per group) and corrected for total protein (mg). Statistically significant differences indicated by [a] $P < 0.05$ vs. Sham control; [b] $P < 0.05$ vs. OVX control.

deparaffinised in xylene, rehydrated in ethanol series, and rinsed in PBS. Endogenous peroxidase activity was quenched by incubation in methanol with 0.3% hydrogen peroxide for 20 min. Sections were incubated with either normal goat (for polyclonal antibodies) or normal horse (for monoclonal antibodies) serum for 30 min before overnight incubation at 4°C with the specific antibodies. They were then incubated with biotinylated goat anti-rabbit (for polyclonal antibodies) or horse anti-mouse (for monoclonal antibodies) IgG for 30 min prior to incubation with peroxidase-labelled avidin-biotin-peroxidase complex (ABC) for 1 h (ABC Elite kit, Vector Laboratories, Peterborough, UK). The immunoreactivity was visualised using a solution of 3,3′diaminobenzidine as chromogen with 0.2% v/v hydrogen peroxide in PBS (for 5 min), to produce a brown end-reaction product, followed by counterstaining with haematoxylin, dehydration, clearing, and mounting. All incubations were carried out at room temperature unless otherwise stated, and washes for 3 × 5 min in PBS were performed after each incubation step except after application of normal sera. Immunocytochemical negative controls included use of nonimmune sera and omission of each of the layers in the ABC sandwich technique.

Statistical analysis

Statistical analyses were performed using the analysis of variance and intergroup comparisons were made using the Dunnett's post hoc test. The data are expressed as mean ± SEM. Intragroup relationships between parameters of tibial failure torque, torsional rigidity, and BMD were assessed using Spearman's two-tailed rank correlation test. A $P <$ 0.05 was regarded as statistically significant.

Results

Morbidity and body weight gain

There were no clinical signs of morbidity in any of the four groups studied except for the weight gain, especially in the ovariectomised control group compared with the Sham controls. This difference occurred despite the fact that the food was restricted to same amount for all groups. Treatment with E2 maintained the body weight at comparable levels with those of the Sham group. Treatment with NG initially reduced the weight gain but the weight at the end of the 28 days treatment period exceeded that of the Sham group (Table 1).

Urine and serum biochemical analyses

There were no differences in urinary deoxypyridinoline excretion except in the ovariectomised rats treated with E2. In this group the deoxypyridinoline excretion was markedly reduced compared with the Sham ($P < 0.05$) and ovariectomised ($P < 0.01$) control groups (Table 2). There were no treatment-related differences in total serum alkaline phosphatase or urinary NO metabolite NO_2^-/NO_3^- excretion (Table 2). However, there was a slight trend toward higher NO_2^-/NO_3^- levels in ovariectomised groups receiving E2 or NG.

Assessment of bone dimensions and mineral density

Tibial gross length and width measurements showed that in both E2 and NG groups the tibiae were shorter than in Sham and OVX groups at the end of the 28-day treatment period (Table 3). However, there were no differences in the tibial width between the groups (Table 3). As expected,

Table 3
Tibial length and width measurements

Parameter	Sham	OVX	OVX + E2	OVX + NG
Tibial length (mm)	37.2 ± 0.30	37.1 ± 0.21	35.2 ± 0.23[c,d]	36.2 ± 0.26[a,b]
Tibial width (mm)	3.7 ± 0.08	3.9 ± 0.08	3.7 ± 0.03	3.9 ± 0.05

Note. Tibial ($n = 7$ per group) length and width measurements were after 28-day treatment period. Statistically significant differences indicated by [a] $P <$ 0.05 vs. Sham control; [b] $P < 0.05$ vs. OVX control; [c] $P < 0.001$ vs Sham control; [d] $P < 0.001$ vs OVX control.

Table 4
Femoral and tibial bone mineral density (BMD) measurements

BMD (mg/cm²)	Sham	OVX	OVX + E2	OVX + NG
Proximal femur	232.9 ± 3.7[b]	225.2 ± 3.7[a]	244.6 ± 2.2[d]	238.7 ± 4.5[b]
Midshaft femur	207.6 ± 2.5	212.3 ± 3.8	204.9 ± 4.6	206.1 ± 3.5
Distal femur	264.5 ± 7.3[d]	239.8 ± 4.8[c]	291.7 ± 2.1[c,d]	260.7 ± 4.3[b]
Proximal tibia	242.4 ± 2.8[d]	211.7 ± 5.4[c]	290.3 ± 3.1[c,d]	231.6 ± 6.2[b]
Midshaft tibia	165.7 ± 3.5	172.1 ± 4.2	162.9 ± 3.0	173.2 ± 3.8
Distal tibia	198.0 ± 3.8	202.9 ± 2.4	194.7 ± 3.8	206.5 ± 3.6

Note. Femoral ($n = 8$ per group) and tibial ($n = 7$ per group) BMD measurements were after 28-day treatment period. Statistically significant differences indicated by [a] $P < 0.05$ vs Sham control; [b] $P < 0.05$ vs OVX control; [c] $P < 0.001$ vs Sham control; [d] $P < 0.001$ vs OVX control.

distal femur and proximal tibia showed the highest BMD response in both the ovariectomised controls and ovariectomised E2 and NG treated groups (Table 4). In ovariectomised controls, there was a clear reduction in BMD at both sites ($P < 0.001$). E2 treatment at 30 μg/kg body weight per day increased the BMD at both sites above that of Sham controls ($P < 0.001$). Twice a day transdermal application of NG ointment (1 mg 2% w/w) prevented the BMD loss in proximal and distal femora and proximal tibia and maintained the BMD levels comparable to those of the Sham controls (Table 4). Fig. 1 shows the mean BMD difference of ovariectomised controls and those treated with E2 or NG compared with the Sham group.

Mechanical properties

There were no differences in tibial failure torque between the Sham and ovariectomised control group, and no change with the E2 and NG treatment group (Table 5). Both E2 and NG supplementation to ovariectomised animals led to a significantly decreased torsional rigidity in comparison to the ovariectomised controls ($P < 0.05$ for E2 and $P < 0.001$ for NG, Table 5). The only significant correlations between the mechanical and BMD parameters were between tibial torsional rigidity and proximal tibial BMD in the E2 group ($r = 0.89$, $P < 0.05$) and tibial failure torque and midshaft tibial BMD in the Sham group ($r = 0.89$, $P < 0.05$).

Immunocytochemistry for nitric oxide synthase isoforms

Neuronal NOS was not detectable in any of the samples studied. Endothelial NOS was not detectable in Sham or ovariectomised rats in mature epiphyseal trabecular osteoblastic lining cells, osteocytes, or osteoclast-like cells but a subpopulation of bone marrow stromal cells expressed eNOS. At the metaphyseal region, eNOS immunoreactivity was observed in mesenchymal osteoblasts-like cells, which lined calcifying cartilage spicules, and in some multinucleated osteoclast-like cells both in Sham and ovariectomised groups (Fig. 2A and B). However, there were no differences in eNOS expression between the Sham and ovariectomised groups. Immunoreactivity for iNOS was detected in some osteoclast-like cells and in a proportion of bone marrow stromal cells in Sham and ovariectomised groups. Inducible NOS was not detectable in epiphyseal osteoblastic lining

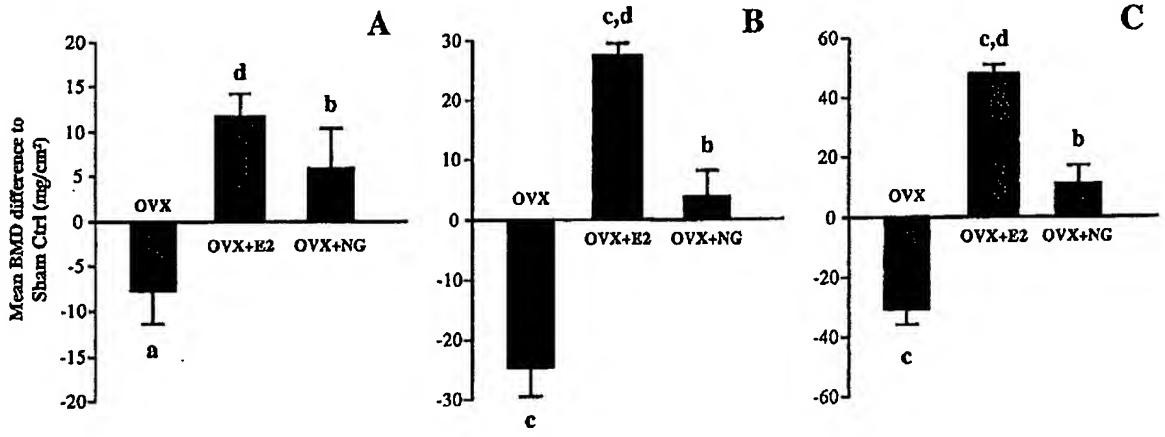

Fig. 1. Mean bone mineral density (BMD) difference of ovariectomised controls (OVX), ovariectomised treated with 17β-estradiol (OVX + E2), and ovariectomised treated with nitroglycerin (OVX + NG) in comparison to Sham group. (A) Proximal femur, (B) distal femur, and (C) proximal tibia. Statistical comparisons were made against Sham controls ([a]$P < 0.05$ and [c]$P < 0.001$) and OVX controls ([b]$P < 0.05$ and [d]$P < 0.001$) (ANOVA).

Table 5
Tibial failure torque and torsional rigidity

Parameter	Sham	OVX	OVX + E2	OVX + NG
Failure torque (0.1 Nm)	1.929 ± 0.137	1.812 ± 0.080	1.996 ± 0.138	2.050 ± 0.127
Torsional rigidity (0.1 Nm/deg)	0.1342 ± 0.0089	0.1639 ± 0.0151	0.1165 ± 0.0080[a]	0.1090 ± 0.0139[b]

Note. Tibial torsional failure torque and torsional rigidity measurements were after 28-day treatment period ($n = 7$ per group). Statistically significant differences indicated by [a] $P < 0.05$ vs. OVX control; [b] $P < 0.001$ vs. OVX control.

cells or osteocytes but in the metaphysis in both Sham and ovariectomised groups it was expressed in osteoblast-like cells associated with calcifying cartilage spicules (Fig. 2C). As for eNOS, there were no detectable differences in iNOS expression between the groups. Fig. 2D shows a negative control obtained by incubation with nonimmune serum.

Discussion

In this study we confirm and extend previous reports on the protective role of NG against estrogen deficiency-induced osteopenia [19–21]. Analyses of skeletal parameters of NOS gene-deficient mice have furnished evidence that both eNOS and cytokine inducible iNOS may contribute to bone metabolism. Some of the protective effects of estrogen on bone seem to be mediated by NO derived from the estrogen responsive eNOS present in all three main bone cell types and in the endothelium of bone vasculature. Young adult rats were chosen for this study as it was speculated that the rapidly growing and remodelling skeleton is more responsive for short-term investigations. Therefore, the results from this short-term investigation do not necessarily reflect those that might have been obtained if a more adult/skeletally mature model would have been chosen.

The results of the present study showed that deoxypyridinoline excretion in the ovariectomised group treated with E2 was markedly reduced in comparison to both the Sham and ovariectomised controls. There were no treatment-related differences in serum total ALP or urinary NO metabolite NO_2^-/NO_3^- excretion. This discrepancy is probably due to the small sample size and high variability. Both E2 and transdermal NG treatment resulted in shorter tibial length in E2 and NG groups at the end of the 28-day treatment period and in comparison to Sham and ovariectomised controls. NG abolished the ovariectomy-induced loss of BMD in the proximal and distal femur and proximal tibia, the regions with high trabecular bone content. In the distal femur and proximal tibia E2 replacement in ovariectomised animals resulted in an increase in BMD above that in Sham controls. There were no significant differences between the groups in tibial torsional failure torque. However, the tibial torsional rigidity was less in the E2 and NG groups than in the ovariectomised controls but not different from those in

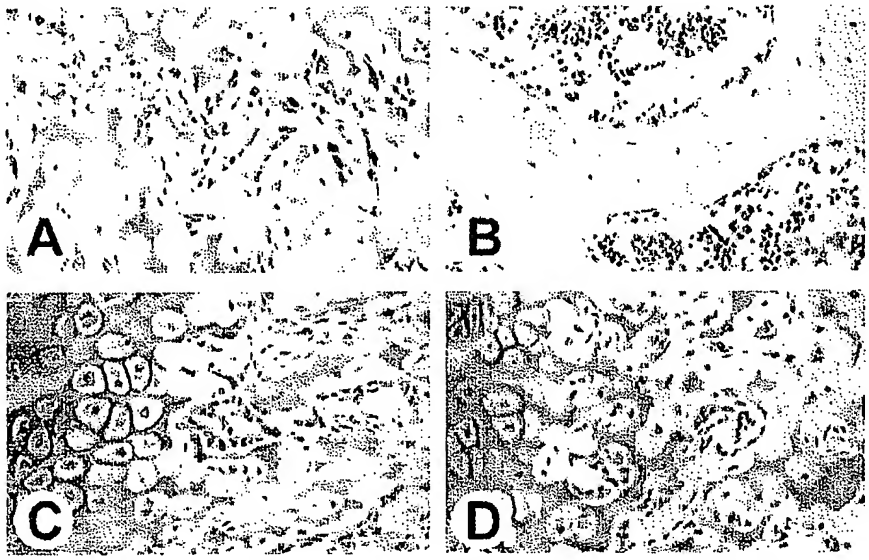

Fig. 2. Endothelial NOS (A and B) and inducible NOS (C) expression in (A) osteoblast-like cells lining calcifying cartilage spicules in the metaphysis and (B) bone marrow stromal cells but not trabecular lining cells in the epiphysis. (C) Inducible NOS was detected in the same regions as eNOS in osteoblast-like cells associated with remodelling and calcifying cartilage. Panel D shows a serial section to that shown in panel C but incubated with nonimmune serum (negative control).

Sham controls. Improvement in the mechanical properties postovariectomy are known to occur due to accelerated bone turnover and a smaller degree of bone maturation and matrix mineralisation [26]. Correlation analysis between the parameters of BMD and mechanical properties showed that tibial torsional rigidity and proximal BMD in the E2 group and tibial failure torque and midshaft tibial BMD in the Sham group showed the only positive correlation. However, the torsional rigidity was similar between the Sham and ovariectomised controls but when comparisons between the E2 and NG treated groups were made against ovariectomised controls the difference became evident. Therefore, it appears that both the E2 and NG treatments maintain the tibial cortical torsional rigidity so that it remains comparable to that of Sham controls. The lack of clear correlation between the BMD and mechanical properties may reflect treatment-related alteration in collagenous matrix synthesis and mineralisation as discussed above [26].

We have previously investigated NOS isoforms and NO mediated vasoreactivity during fracture repair. In these experiments, a stabilised tibial fracture model in Wistar rats was used. Western blots and immunolocalisation showed an elevation in eNOS at Day 1 after fracture that was maintained throughout the 28-day study period. Calcium-dependent NOS activity was similarly elevated at Day 1 after fracture. In contrast, iNOS synthesis was initially reduced before returning back to control levels [27]. Laser Doppler flowmetry showed high vascular reactivity at the fracture site at Day 1 after fracture, as a response to bolus intravenous administration of *N*-nitro-L-arginine methyl ester (1–100 μmol/kg) compared to other time points assessed (0–28 days) or to the contralateral intact limb. These results imply that restoration of blood flow at the fracture site is regulated by an NO-dependent mechanism which in turn affects the preexisting vessels [28]. These results further suggest that the vascular response of bone may be a significant factor that contributes to the protective effect of NG against osteopenia. Continuous administration of NG and other NO donating organic nitrates can lead to vascular and haemodynamic tolerance [29]. In a recent study on ovariectomy-induced osteopenia in rats, the beneficial effects of NG on bone turnover and BMD decreased with increasing dosage frequency [20]. In keeping with this, in a prospective population study, elderly women who daily used NG, isosorbide monodinitrate, or isosorbide dinitrate, had slightly greater hip BMD but no difference in heel BMD in comparison to nonusers of nitrates. In contrast, women who used nitrate compounds only intermittently had substantially greater hip and heel BMD [30].

Studies on NOS gene-deficient mice provide insight on the functional significance and the relative contributions that the different NOS isoforms and NO may exert on skeletal remodelling. Histomorphometric and BMD analyses of young adult (6–9 week-old) eNOS gene-deficient mice reveal marked abnormalities in bone volume and formation rate and reduced BMD that are mainly related to dysfunctional osteoblasts [17]. Ovariectomised eNOS gene-deficient mice loose bone comparable to that observed in wild-type controls but show a blunted anabolic response to high dose of estrogen [18]. Endothelial NOS protein and mRNA levels also seem to depend on the stage of rat skeletal maturation so that they are highly expressed in neonates and during rapid postnatal growth and at sites of remodelling [25].

Induction of endogenous NO generation by estrogen, mechanical strain, and shear stress has been suggested as a mechanism for modulation and coupling of bone cell functions to both systemic and local factors [5–11]. In the present study, we found no evidence of the presence of nNOS protein in Sham or ovariectomised groups, but both eNOS and iNOS were predominantly detected in metaphyseal osteoblasts associated with calcifying cartilaginous spicules. However, there were no clear differences in eNOS or iNOS expression between the Sham and ovariectomised groups. Nevertheless, the presence of these NOS isoforms point toward a physiological and local role for NO that is likely to include tonic restrain of osteoclast activity [31–33] and enhanced osteoblast activity leading to deposition of new mineralised bone matrix. In vitro experiments have shown that eNOS gene-deficient osteoblasts exhibit an ablated anabolic response to E2 in terms of thymidine incorporation, ALP activity, and mineralised bone nodule formation [6,17,18]. However, the relative contributions of vascular and bone cell function as a response to transdermal NG (or subcutaneous E2) and in terms of clear changes in BMD that accompany such treatment still remain unclear.

Taken together, these data confirm that transdermal delivery of the NO donor NG counteracts the development of skeletal osteopenia associated with ovariectomy-induced estrogen deficiency. The effect of transdermal NG on bone metabolism is likely to include both a local bone cell-mediated and vasoreactive response of the bone. We found no signs of gross morbidity in any of the four groups except for the rapid weight gain in the ovariectomised control group and the initial slow weight gain in the ovariectomised NG treated group. However, development of vascular tolerance to NG was not addressed and should be considered in future investigations. Nevertheless, the data confirm and extend the suggestion that systemic administration of organic nitrate NG may be beneficial in conditions where bone turnover is compromised such as in estrogen deficiency-induced osteopenia.

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